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The fate of chemoresistance in triple negative breast cancer (TNBC)

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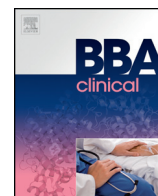
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The fate of chemoresistance in triple negative breast cancer (TNBC)



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ABSTRACT

Background: Treatment options for women presenting with triple negative breast cancer (TNBC) are limited due to the lack of a therapeutic target and as a result, are managed with standard chemotherapy such as paclitaxel (Taxol®).

Following chemotherapy, the ideal tumour response is apoptotic cell death. Post-chemotherapy, cells can maintain viability by undergoing viable cellular responses such as cellular senescence, generating secretomes which can directly enhance the malignant phenotype.

Scope of Review: How tumour cells retain viability in response to chemotherapeutic engagement is discussed. In addition we discuss the implications of this retained tumour cell viability in the context of the development of recurrent and metastatic TNBC disease.

Current adjuvant and neo-adjuvant treatments available and the novel potential therapies that are being researched are also reviewed.

Major conclusions: Cellular senescence and cytoprotective autophagy are potential mechanisms of chemoresistance in TNBC. These two non-apoptotic outcomes in response to chemotherapy are inextricably linked and are neglected outcomes of investigation in the chemotherapeutic arena. Cellular fate assessments may therefore have the potential to predict TNBC patient outcome.

General Significance: Focusing on the fact that cancer cells can bypass the desired cellular apoptotic response to chemotherapy through cellular senescence and cytoprotective autophagy will highlight the importance of targeting non-apoptotic survival pathways to enhance chemotherapeutic efficacy.

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1. Introduction

Triple negative breast cancers (TNBCs) are a specific subtype of epithelial breast tumours that are immunohistochemically negative for the protein expression of the oestrogen receptor (ER), the progesterone receptor (PR) and lack overexpression/gene amplification of HER2 [1].

Approximately, 10–14% of breast cancers are triple negative and the methods used to perform a diagnosis generally dictate the frequency [2]. Pre-analytic and analytic issues such as delay from tissue collection to fixation (risk of cold ischemia) and assay reproducibility (assay validation and standardisation) can impact on whether a tumour is assigned as Triple Negative or not [3].

1.1. Immunohistochemical testing

The characterisation and measurement of the oestrogen receptor (ER) protein was first described by Jensen et al., in 1967 and in 1973, McGuire et al., described the dextran-coated charcoal (DCC) biochemical assay to quantify the ER protein [4,5]. It was also then postulated that detection of the oestrogen receptor may predict response to endocrine therapy. Immunohistochemical (IHC) methodologies have been used since the early 1990s and remain by far the most common type of assay worldwide. However, there is an inadequate standardisation of the IHC methodology. A recent “ring study” showed an initial ER testing concordance between two central laboratories at just 85%, which was fully resolved once both laboratories adopted the same ER assay methods [6].

Recent misclassification (false negative) reports have raised awareness concerning the limitations of immunohistochemistry (IHC) in the assessment of the oestrogen receptor (ER) in breast cancer. Hede et al. (2008) describe the incident in Canada where there was a 40% misclassification rate between local and central laboratories highlighting the current limitations associated with ER protein testing [7].

Rimm et al., 2011 describe a potential method for standardising ER measurement “on a slide” using a quantitative immunofluorescence (QIF) assay [8]. Moreover, Rimm's data also show that an assessment of the intensity threshold by using this assay on two independent cohorts resulted in discordance in the 10% to 20% range with current IHC methods. Overall, the findings of the study were that (a) the threshold of immunoreactivity appears to be more important than the percentage positivity in the generation of discordant or false-negative assays and that (b) the standardisation method by using the QIF assay appeared to be more sensitive than the traditional IHC assay. This was despite the fact that the same antibody was used for the detection of ER (1D5). Rimm et al., concluded that (a) intensity threshold, in other words, what actually constitutes a “positive” nucleus is the key determinant of variable scoring that needs to be controlled to correctly define ER characterisation and that (b) the QIF assay is more sensitive than routine IHC [8].

The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) issued guidelines to address this issue of discordance in ER assessment. An IHC cutoff of 1% positive nuclei is now used to define a tumour as ER positive compared to the previous cut off of 10% positive nuclei [9]. Few ER-positive tumours have true IHC staining between 1% and 10% [10]. However, false-negative ER results can still occur even when using a validated and reproducible IHC assay and an imaging analysis system is unlikely to improve detection of tumours with low levels of ER. This justifies the use of a low IHC cutoff (1%) for clinical use to minimise this risk of false-negative ER. In the absence of a linear correlation between ER expression by IHC and clinical outcome, available data suggest that a qualitative assessment (ER positive v ER negative) is more critical than a quantitative IHC assay [3].

Accurate testing for the HER2/neu gene overexpression is also an issue where false-positives using IHC can occur. In 1999, Jacobs et al., highlighted this with the HercepTest, where tumours identified as

HER2 positive by this test were subsequently described as negative using FISH and other IHC methods using the same antibody [11]. The "gold standard" used to identify these false-positive results has been tested for HER2/neu gene amplification by FISH. This is due to the greater specificity and sensitivity of FISH when either test is compared with HER2/neu overexpression as determined by Northern and Western blot analyses [12,13].

Studies have also shown that the problem of false-positive HER2 results, involve IHC techniques most significantly when the result is 2+ [14]. Specifically, as few as 17% of 2+ HercepTest carcinomas demonstrate gene amplification by FISH [15]. The overwhelming majority of 3+ positives demonstrate HER2 gene amplification. However, in a study by Mass et al., 2000, 11% of 3+ positive tumours did not demonstrate gene amplification by FISH. This indicates that false-positive results are a potential problem in this group as well [16]. False-negative results, in other words, IHC-negative/FISH-positive carcinomas, are rare in the 0–1+ IHC groupings.

The ASCO and the College of American Pathologists (CAP) recently updated HER2 testing guidelines to improve the accuracy of HER2 testing and its utility as a predictive marker in invasive breast cancer [3]. A systematic review was conducted to identify areas requiring clarification and to improve the accuracy of HER2 testing by immunohistochemistry (IHC) or *in situ* hybridisation (ISH). The guidelines have been published jointly in the Journal of Clinical Oncology and the Archives of Pathology & Laboratory Medicine. These recommendations will improve the analytical validity of HER2 testing, its clinical utility and the communication among health-care providers [17].

Recommendations include:

- That HER2 status (HER2 negative or positive) be determined in all patients with invasive (early stage or recurrence) breast cancer on the basis of one or more HER2 test results (negative, equivocal, or positive).
- That testing criteria define HER2-positive status when there is evidence of protein overexpression (IHC) or gene amplification (HER2 copy number or HER2/CEP17 ratio by ISH based on counting at least 20 cells within the area).
- That if results are equivocal (revised criteria) reflex testing should be performed using an alternative assay (IHC or ISH).
- That repeat testing should be considered if results seem discordant with other histopathological findings.
- Those laboratories should demonstrate high concordance with a validated HER2 test on a sufficiently large and representative set of specimens.
- That HER2 testing must be performed in a laboratory accredited by CAP or another accrediting entity.

1.2. Epidemiology

TNBC's are typically of the histological ductal type with a high mitotic rate, increased lymphocytic infiltrate, high grade and large tumour size. In addition, TNBC patients commonly present with early visceral metastases and have lymph node involvement at the time of diagnosis [18,19]. Approximately 15% of TNBC patients will develop brain metastases with autopsy studies reporting a 30% rate [20]. This aggressive metastatic cancer contributes to the overall shortened survival of patients with TNBC [21].

In relation to age, TNBC's affect younger women more frequently and are more prevalent in African-Americans who are three times more likely to present with TNBC than Caucasian women [22]. In fact 39% of all African American premenopausal women diagnosed with breast cancer are diagnosed with TNBC [23]. The prevalence of TNBC in this same age group in non-African American women is demonstrably less approximating at 15%. These ethnic and/or menopausal differences are not seen in either, the ER+/HER2+ breast cancer subgroup or the ER+/HER2- subgroup [23]. Women presenting with

TNBC also have a higher rate of distant recurrence and a poorer prognosis than women with other subtypes of breast cancer. Paradoxically, this higher rate of distant recurrence occurs despite these women having initial higher response rates to neoadjuvant chemotherapy [24].

1.3. TNBC molecular and gene expression subtypes; a heterogeneous cancer

As a result of gene expression profiling, breast cancer has been classified into five distinct molecular subtypes, namely; normal-like, basal, luminal A & B and HER-2 enriched [25]. A *Claudin-low* sub-type is another described molecular subtype referring to tumours showing features of mesenchymal and mammary stem cells [26]. Interestingly, tumour cells that survive chemotherapy show features similar to mesenchymal *claudin-low* tumour cells [27].

In relation to TNBC's, 75% fall into the basal-subtype and express genes normally associated with normal basal like myoepithelial cells of the breast ductal and lobular system, such as the epidermal growth factor receptor (EGFR) [28]. Basal-like breast cancers tend to express cytokeratins associated with basal types of cancers, as they arise from the outer basal layer. It has also been reported that almost 82% of basal-like breast cancers express p53 compared with 13% in the luminal A subgroup [29]. Useful immunohistochemical markers for characterising basal like carcinomas include CK5, CK6, CK14, CK8/CK18, p63, P-cadherin, vimentin, epidermal growth factor receptor 1 (EGFR1), c-kit, and other growth factors such as vascular-endothelial growth factor (VEGF) and the insulin-like growth factor receptor (IGFR) [28].

Lehmann et al., 2011 have analysed the gene-expression profiles from 21 breast cancer data sets of which 587 were TNBC [30]. Six TNBC subtypes were shown to have unique gene expression profiles, namely basal-like (BL1 & BL20), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL) and a luminal androgen receptor (LAR) subtype. Identification of TNBC cell line models representative of these subtypes was performed using further gene expression (GE) analysis. Predicted "driver" signalling pathways were pharmacologically targeted in these cell line models as proof of concept that the analysis of distinct GE signatures had the potential to inform patient therapy decision.

In general, the BL1 and BL2 TNBC subtypes had higher expression of cell cycle and DNA damage response genes. Moreover, representative cell lines preferentially responded to cisplatin. In the M and MSL subtypes, these were enriched in GE profiles representing the epithelial-mesenchymal transition (EMT), and growth factor pathways with cell models responding to NVP-BE235 (a PI3K/mTOR inhibitor) and dasatinib (an *abl/src* inhibitor). The LAR subtype included patients with a decreased relapse-free survival and was characterised by androgen receptor (AR) signalling. LAR cell lines were uniquely sensitive to the AR antagonist bicalutamide. To the future, the identification and characterisation of these diverse TNBC subtypes and the molecular drivers in corresponding cell line models has the potential to provide great insight into the heterogeneity of this disease and to provide preclinical platforms for the development of effective treatment.

2. Adjuvant & neoadjuvant chemotherapy used in TNBC

It is well established that TNBC is an aggressive group of breast cancer subtypes despite having a good initial response to chemotherapy. It is also well established that patients with residual TNBC disease post neo-adjuvant chemotherapy have a worse prognosis than those presenting with non-TNBC [2]. Importantly, there is no preferred standard chemotherapy for these patients and typically tumour size, lymph node status, grade, overall performance status and the presence or absence of medical co-morbidities will determine the regimens used. For women presenting with triple negative breast cancer (TNBC), it is established that in the absence of ER, PR and HER-2, endocrine therapies such as Tamoxifen and aromatase inhibitors and HER-2 directed therapies such as Trastuzumab and Lapatinib are not efficacious. Triple negative breast

cancers seem to be particularly chemo-sensitive to anthracyclines and taxanes which are part of the standard therapy used for high risk patients, for example those patients with node positive disease. In spite of this, however, there is an overall poorer survival [31].

Although TNBCs are generally very susceptible to chemotherapy initially, early complete response (CR) does not correlate with overall survival. Specifically, the risk of relapse for TNBC patients in the first 3–5 years is significantly higher than for women presenting with hormone positive breast cancer [32,33]. Clinically, this makes it particularly challenging to find the optimal chemotherapy which will result in a longer metastasis free and overall survival for these women.

Neoadjuvant chemotherapy is clinically where most of the knowledge about chemosensitivity has arisen. Specifically, there is a higher pathological complete response, (pCR) related to neoadjuvant anthracycline based chemotherapy in TNBC compared to luminal non-TNBC subtypes. However, despite initial chemosensitivity, metastatic relapse paradoxically appears to occur at higher rates for TNBC tumours [2].

In the adjuvant therapy space, the principles for nonTNBC apply equally to TNBC. These therefore can include:-

- 1) Doxorubicin or Epirubicin (Anthracyclines)
- 2) AC (Doxorubicin and Cyclophosphamide)
- 3) Paclitaxel and Docetaxel (often weekly and often every third week respectively), frequently used in combination with Cyclophosphamide or 5-Fluorouracil. For example, ACT (doxorubicin, cyclophosphamide, paclitaxel), TC (docetaxel and cyclophosphamide), TFEC (docetaxel, 5-Fluorouracil, epirubicin, cyclophosphamide)
- 4) CMF (cyclophosphamide, methotrexate, 5-Fluorouracil)
- 5) Antimetabolites such as Gemcitabine or Capecitabine and other microtubule inhibitors or stabilisers such as Vinorelbine.
- 6) Non-taxane anti-tubulin agents such as Eribulin and Ixabepilone, which are associated with limited clinical efficacy in TNBC as opposed to non-TNBC presentations.

3. Novel potential therapies for TNBC

TNBCs can arise in *BRCA1* mutation carriers and have gene expression profiles similar to those of *BRCA1*-deficient tumours [19]. Moreover, *BRCA1*-associated breast cancer appears to cluster in the basal-like subtype [34]. *BRCA1* mutation carriers often display basal like gene expression profiles, and there is increasing evidence that a TNBC basal like subtype develops mainly through a *BRCA1*-related pathway, resulting in increased genomic instability [35]. For *BRCA1* mutation carriers, the incidence of TNBC approximates to 70% [36,37], although the incidence of *BRCA* mutations in TNBC can vary from 16% to 42% [38].

Even though a patient may lack the *BRCA-1* somatic mutation, sporadically arising basal-like cancers often display a dysfunctional *BRCA-1* pathway [39]. TNBC's and *BRCA1*-deficient tumours share certain histological features including genomic instability, DNA repair defects [39] and mutations in p53, which disrupt apoptosis and which are associated with a poor prognosis [40]. Importantly, *BRCA1* plays an important role in DNA double-strand break repair, contributing to the maintenance of DNA stability [41].

3.1. Poly ADP-ribose polymerase (PARP) inhibition

Poly ADP-ribose polymerase (PARP) enzymes are critical for the appropriate processing and repair of DNA breaks [42]. Tumour cell lines lacking functional *BRCA1* or *BRCA2* are sensitive to PARP inhibitors in preclinical studies [43]. Clinical trials using both PARP inhibitors and DNA-damaging agents in TNBC have shown some promising results in *BRCA1/2*-mutant tumours [44]. Specifically, the PARP inhibitor *olaparib* has been used in the treatment of patients with *BRCA 1* and 2 deficient tumours: — cancers which share similar features to sporadic TNBC, where some efficacy has been shown in a Phase II trial in *BRCA*-

deficient recurrent advanced breast cancers, with enrolled patients having previously been given three previous chemotherapy regimens prior to the trial. Clinically, the objective response rates were 41% ($n = 11$) and 22% ($n = 6$) for patients receiving *olaparib* 400 mg twice daily and *olaparib* 100 mg twice daily, respectively. Moreover, the median progression-free survival in the 400 mg BD cohort was 5.7 months, compared with 3.8 months in the 100 mg twice daily cohort [45, 46]. However, clinical data from another similarly sized cohort of tumours ($n = 15$) failed to demonstrate any response in *BRCA* mutation-negative TNBC patients [47].

The PARP inhibitor *iniparib* versus placebo with Gemcitabine and Carboplatin has demonstrated significant improvement in patients with pre-treated TNBC with an overall response rate (ORR) of 52% versus 33%, progression free survival (PFS) of 5.9 versus 3.6 months and overall survival (OS) of 12.3 versus 7.7 months [48]. However, results of a multicentre, phase III trial assessing the same *iniparib* combination in advanced TNBC failed to meet the primary study end points with a PFS of 4.1 versus 5.1 months and overall survival of 11.1 versus 11.8 months [49].

The combination of *Veliparib* with the oral alkylating agent temozolamide, resulted in an improved response, bearing in mind that 20% of the patients in this trial had *BRCA* germ line mutations. However, this Phase II trial did not include a TNBC subgroup analysis [50].

Overall, PARP inhibitors have shown little benefit in unselected TNBC populations, either as a single regimen [46] or used in combination with other standard chemotherapeutics [51,52], although *BRCA*-selected patient groups may potentially benefit from ongoing PARP development [53].

3.2. Platinum salts

Three randomised trials have investigated the clinical efficacy of using platinum based regimens for the treatment of TNBC, [54–56] with further randomised trials evaluating the addition of platinum derivatives to standard adjuvant therapy (NCT01150513 & NCT01216111). NCR01378533 is an adjuvant phase III trial comparing a dose dense anthracycline–taxane regimen to dose dense paclitaxel and carboplatin. The combination of a platinum based agent with the PARP inhibitor PF-01367338 is also currently being evaluated following preoperative chemotherapy in patients with TNBC or *BRCA1/2*-associated BC (NCT01074970).

In the metastatic and second line setting, the addition of cisplatin to metronomic methotrexate and cyclophosphamide has resulted in an improvement in the median time-to-progression (TTP) of 6 months (from 7 to 13 months) and OS of 4 months (from 12 to 16 months) [54].

Carey et al., 2008 have also shown that when carboplatin is added to the single-agent cetuximab in pretreated advanced TNBC patients, the overall response rate (ORR) is 17% versus 6% [55]. Cetuximab plus carboplatin produced partial responses in 71% (13/18) patients compared to cetuximab monotherapy; 2 (6) of 31% patients and stable disease lasting ≥ 6 months in 6 additional patients (9%) [57].

However, the addition of cisplatin to the anthracycline–taxane based regimen showed no improvement in pathological complete response (pCR) rates or rates of breast conserving surgery in the basal like TNBC cohort of patients enrolled in The Spanish Breast Cancer Research Group (GEICAM) 2006 phase II RCT [56].

In phase II trials [56,58], the combination of weekly cisplatin added to weekly epirubicin and paclitaxel, produced a pCR rate of 62% and 5-year disease-free survival (DFS) and OS values of 76% and 89%, respectively. In addition, Liu et al., 2013 have undertaken a meta analysis of platinum-based chemotherapy in TNBC, which included 7 studies and 717 patients of which 31% were TNBC [59]. These results showed that in the neo-adjuvant setting, the clinical complete response (cCR) rate and the pathological complete response (pCR) rates were significantly higher for the TNBC group compared with the non-TNBC group (OR, 2.68; 95% CI, 1.69–6.57; $p = 0.03$ and OR, 2.89; 95% CI, 1.28, 6.53;

$p = 0.01$, respectively). However, in advanced/metastatic breast cancers, the cCR, partial response (PR) and the disease control rates for the TNBC group were not significantly different compared with the non-TNBC group. In the same study [59], the 6-month progression-free survival (PFS) rate for the TNBC group was higher than that of the non-TNBC group in all patients (OR, 1.81; 95% CI, 1.11–2.96; $P = 0.02$). Nonetheless, the 1- and 2-year PFS rates were not significantly different between the TNBC and non-TNBC group (OR, 1.42; 95% CI, 0.69–2.92; $P = 0.35$ and OR, 1.11; 95% CI, 0.35–3.52; $P = 0.85$, respectively). Also, there was no significant difference in the PFS rates between the groups in patients with advanced/metastatic breast cancer.

In summary, this meta-analysis demonstrated that in the neoadjuvant setting, platinum-based chemotherapy in the TNBC group shows short-term efficacy compared with the non-TNBC group. Unfortunately, no improvement in advanced TNBC was demonstrated [59].

The addition of platinum agents to anthracycline and/or taxane regimens in the neoadjuvant setting has shown promising outcomes, with pathological pCRs ranging from 30% to 62%. The results of the Phase II CALGB/Alliance 40603 clinical trial which looked at the impact of the addition of carboplatin and/or bevacizumab to neoadjuvant weekly paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pCR rates in TNBC were presented at the 2013 San Antonio Breast Cancer symposium [60]. The addition of carboplatin to standard neoadjuvant chemotherapy increased pCR. Despite increases in pCR with bevacizumab, side effects were more concerning with this drug.

Pathologic complete response rates were 60% for patients receiving carboplatin and 46% in patients who did not receive carboplatin; an increase of 76% ($P = .0018$). Defined by no disease in the breast or axilla, pCR rates increased to 54% and 41%, respectively; a 71% increase ($P = .0029$).

The addition of bevacizumab was also associated with an improvement in pCR in the breast only, producing pCRs in 59% of patients receiving the drug and 48% of patients not treated with bevacizumab; a 58% increase ($P = 0.0089$). Pathologic complete response rates for both breast and axilla were 52% and 44%, respectively; a 36% nonsignificant increase ($P = .0570$). When carboplatin and bevacizumab were used in combination, the highest pCR rate of 67% was achieved. However, the P value for the carboplatin/bevacizumab interaction was .52, indicating a lack of a synergistic effect.

3.3. Microtubule stabilising agents

A review in 2012, focused on potential new treatments for TNBC and included the microtubule-stabilising agent, ixabepilone [61]. Clinical trials have shown that when used with capecitabine (a second-line therapy widely used in anthracycline and taxane-resistant disease), ixabepilone has an acceptable safety profile and clinical activity, when compared with capecitabine alone. The women in this trial had locally advanced or metastatic cancer, pre-treated with an anthracycline and a taxane [62,63]. Analysis of pooled data from these trials found that the overall response rate (ORR) (31 vs. 15%) and progression free survival (PFS) (4.2 vs. 1.7 months), were improved for women with TNBC who received combination therapy of ixabepilone and capecitabine as opposed to those women who received single-agent capecitabine alone [64].

3.4. Angiogenesis inhibitors

Tumour VEGF expression is significantly higher in TNBC compared with non TNBC presentations [65]. Studies identifying potential molecular markers of TNBC, such as VEGF [66], EGFR, Tyrosine Kinases [67], Src [68], and mTOR [69], have impacted on the design of clinical trials, investigating targeted treatments in the space of angiogenesis inhibitors.

3.4.1. Anti-VEGF monoclonal antibody; bevacizumab

Bevacizumab, the anti-VEGF agent (Trade name Avastin® Genentech/Roche), has received controversial attention despite various promising

results from a variety of clinical trials. Specifically, there are multiple Phase III trials looking at the efficacy of bevacizumab in breast cancer, with three trials assessing the drug as a first line agent along with chemotherapy in the TNBC metastatic setting. These three trials are (1) the E2100 study, (2) the Avastin & Docetaxel (AVADO) trial and (3) the Regimens in Bevacizumab for Breast Oncology (RiBBOn)-1 trial [70].

In the E2100 trial, the risk of progression in first-line TNBC patients was reduced by 51% whilst the median PFS doubled (5.3 versus 10.6 months), when bevacizumab was added to paclitaxel. The AVADO trial demonstrated a 47% reduction in disease progression when bevacizumab was added to docetaxel in the TNBC subgroup. However, there did not appear to be any clear benefit in the RiBBOn-1 trial when bevacizumab was added to chemotherapy regimens.

Similarly, on TNBC subgroup analysis ($N = 159$), patient improvements were observed in the second line setting only in the phase III RiBBOn-2 trial, demonstrating a 51% reduced risk of disease progression and a doubling of median PFS among women treated with the bevacizumab combination, compared with women treated with chemotherapy alone [2.7 versus 6.0 months; HR = 0.49 (95% CI 0.33–0.74), $P = 0.0006$]. There was also a trend towards improved survival [median, 17.9 versus 12.6 months; HR = 0.624 (95% CI 0.39–1.007), $P = 0.0534$].

In the neo-adjuvant setting, two studies addressing the role of bevacizumab are the GeparQuinto [71] and the National Surgical Adjuvant Breast and Bowel Project 40 (NSABP)-B40 [72] trials, where this anti-VEGF agent was combined with neoadjuvant anthracycline–taxane chemotherapy. In the TNBC subgroup, the two studies showed the opposing results. Specifically, GeparQuinto demonstrated a statistically significant improvement in pCR rates ($N = 663$; 39.3% versus 27.9%, $P = 0.003$) for patients receiving bevacizumab compared with chemotherapy while, The National Surgical Adjuvant Breast and Bowel Project (NSABP)-B40 showed no improvement of pCR rates. ($N = 479$; 51.3% versus 47.3%, $P = 0.44$). The differing clinical outputs in the results from these two trials might possibly reflect variations in how HER2-negativity was defined in the patient groups treated in the trials and in the therapy regimens they received [73]. Longterm DFS and OS results are not yet available for these two studies. However, the potential use of these anti-VEGF drugs in the treatment of early TNBC will become clearer as further results become available from the GeparQuinto and (NSABP)-B40 in addition to the ongoing Bevacizumab Adjuvant Therapy in Triple negative Breast Cancer (BEATRICE) Phase III trial.

In 2010, the FDA re-addressed the efficacy of the drug, Avastin® (bevacizumab) for the treatment of women with breast cancer, due to its reported side effects/safety concerns such as thromboembolic events, gastrointestinal perforation, wound healing complications, haemorrhage, hypertensive crisis, nephrotic syndrome, congestive heart failure and neutropenic sepsis. The FDA gradually removed breast cancer for the use of Avastin® due to these concerns and on November 18, 2011, the Food and Drug Administration (FDA) Commissioner, Margaret Hamburg revoked the agency's accelerated approval of the breast cancer indication for bevacizumab (Avastin® (bevacizumab) made by Genentech). The Oncology Drugs Advisory Committee rejected Genentech's appeal based in part on the modest risk-benefit ratio of this agent in the overall population [74].

In 2011, Ranpura et al., published a meta-analysis in JAMA, highlighting the adverse side effects of bevacizumab, showing that when added to chemotherapy regimens, particular taxane and platinum drugs. Specifically bevacizumab was associated with an increased risk of fatal adverse events (FAEs), compared to using chemotherapy alone [75]. Moreover, a total of 10,217 patients with a variety of advanced solid tumours from 16 randomised controlled trials (RCTs) were included in the analyses. The overall incidence of fatal adverse events with bevacizumab was 2.5% (95% CI, 1.7%–3.9%), with haemorrhage (23.5%), neutropenia (12.2%), and gastrointestinal tract perforation (7.1%), being the most common. Bevacizumab used for metastatic breast cancer has also not been shown to provide a patient benefit in

terms of delay in tumour growth that would justify its serious and potentially life-threatening risks. Therefore, there is currently no evidence that the use of bevacizumab will either help women with breast cancer live longer or improve their quality of life. This decision involves bevacizumab's use in combination with paclitaxel for patients who have not been treated with chemotherapy for HER2 negative metastatic breast cancer. This indication has now been removed from bevacizumab's product labelling.

3.5. Epithelial growth factor receptor (EGFR) inhibitors

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4).

3.5.1. Anti-EGFR monoclonal antibody; cetuximab

In patients with metastatic TNBC, a cetuximab plus cisplatin combination (BALI-I Trial) has demonstrated an overall better response rate of 20% when compared to a 10% overall better response rate with cisplatin alone [76]. Cisplatin plus cetuximab also resulted in longer PFS compared with cisplatin alone (median, 3.7 v 1.5 months) with a corresponding median OS of 12.9 versus 9.4 months. Common grade 3/4 adverse events included acne-like rash, neutropenia, and fatigue. However, the BALI-I trial failed to reach its primary endpoint, namely the overall response rate, despite apparently doubling it, compared to the single regimen. This combination also appears to lengthen PFS and OS. There was also a concern of toxicity such as diarrhoea when cetuximab was added to chemotherapy such as carboplatin and irinotecan despite an increased overall response rate in the TNBC subset of O'Shaughnessy's phase II trial [77]. Analysis of two randomised trials has shown that basal like breast cancer patients lacking PTEN and alpha basic crystalline, preferentially respond to cetuximab treatment [78]. Therefore, the potential efficacy of anti-EGFR strategies needs further investigation with further trials such as those investigating combinations of cetuximab with the non-taxane anti-tubulin agent, ixabepilone in both the early (NCT01097642) and advanced (NCT00633464) settings.

3.5.2. EGF/Src tyrosine kinase inhibitors

Of the multiple-tyrosine kinase inhibitors, dasatinib, sunitinib and neratinib have been studied in a setting where patients have already previously received heavy doses of standard chemotherapy [79]. Dasatinib, which has activity in haematology malignancies and prostate cancer, has been shown to inhibit the growth of TNBC cell lines *in vitro* when used in combination with standard chemotherapy such as cisplatin and when used as a single agent [68]. However, a Phase II clinical trial of women with locally advanced or metastatic TNBC showed a clinical benefit rate of only 9.3% when dasatinib was used as a single agent [80].

In relation to sunitinib, this tyrosine kinase inhibitor was not recommended to be used in combination with doxorubicin in patients with newly diagnosed HER2 negative advanced or in patients with metastatic breast cancer following a Phase III trial [81]. Like bevacizumab, sunitinib is increasingly regarded as being ineffective in breast cancer [82].

For neratinib, the NCT0111825 Phase I/II trial is currently investigating its efficacy in combination with the mTOR inhibitor temsirolimus, both in the metastatic TNBC and metastatic HER2 amplified setting. The primary outcome is to estimate the maximum tolerated dose with safety and efficacy as secondary outcomes. Recruitment is through the Memorial Sloan-Kettering Cancer Centre group and December 2015 is the estimated completion for this trial.

The tyrosine kinase phosphatase, PTPN12, acts by inhibiting multiple oncogenic tyrosine kinases, including HER2 and EGFR and appears to be mutated in 5% of TNBCs and absent from as many as 60% [83]. PTPN12 exhibits tumour suppressor actions when reintroduced into breast cancer cells devoid of the enzyme [83]. Sunitinib and lapatinib might therefore have beneficial activity against these tumours. Phase 1/2

clinical trials should commence to determine whether FDA approved tyrosine kinase inhibitors are effective in patients with PTPN12-deficient TNBC tumours. The possibility of a clinical trial with sunitinib and crizotinib, two drugs that are FDA-approved and marketed by Pfizer is being explored by the Stand up to Cancer Breast Cancer Dream Team.

3.6. mTOR inhibitors

The serine-threonine kinase mammalian target of rapamycin (mTOR) promotes protein translation, angiogenesis, proliferation and migration [84]. Moreover, inhibiting mTOR's mediated PI3K/Akt signalling pathway abolishes cellular proliferative responses and causes cell cycle arrest. As PI3K/Akt overactivity has been identified in a number of breast cancers [69], rapamycin and its analogs temsirolimus, everolimus, and deforolimus, are undergoing clinical evaluation in TNBC treatment [85]. The mesenchymal subgroup of TNBC may benefit from a treatment targeting and inhibiting mTOR as the aggressive metaplastic and claudin-low type is enriched in epithelial-mesenchymal transition and stem-cell like features which make them more chemoresistant than the luminal & basal subtypes [86].

In relation to the efficacy of everolimus, the addition of this mTOR inhibitor to standard chemotherapy did not significantly improve pathological pCR rates in a phase II neoadjuvant trial of 50 TNBC patients [87] with another neoadjuvant trial in progress (NCT00930930). Ongoing studies investigating the use of everolimus in the treatment of advanced TNBC are being investigated in the NCT01272141, NCT0111825 and NCT00827567 trials. Specifically, NCT01272141 is looking at the combination of lapatinib, a dual role HER2/EGFR inhibitor and everolimus in locally advanced or metastatic TNBC. NCT0111825 is addressing the role of temsirolimus plus neratinib in patients with either metastatic HER2-amplified or TNBC, while NCT00827567 is examining the use of everolimus as a single agent in metastatic TNBC.

The PTEN phosphatase, which inhibits the pro-growth PI3K/Akt pathway, is lost in the majority of TNBC tumours, particularly African-American and Hispanic patients [88]. PTEN loss is associated with reduced disease-free survival, but might also render these tumours susceptible to PI3K/Akt inhibitors.

The TNBC cell line MDA-MB 435 with PTEN deficiency has shown increased sensitivity to mTOR inhibition [89], while Steelman et al., 2008 also demonstrated that suppression of PTEN function in the MCF 7 non-TNBC breast cancer cell line, also increased sensitivity to the mTOR inhibitor rapamycin [90]. Overexpression of S6K1 and expression of phosphorylated Akt could be predictors of rapamycin sensitivity in breast cancer patients with changes in cyclin D1 levels providing a potential pharmacodynamic marker of response to rapamycin [91].

3.7. Androgen inhibition

Gucalp et al., 2010 have shown that androgens, through stimulating the androgen receptor can induce proliferative changes in breast cancer cell lines and promote tumourigenesis in animal models [92]. Androgen-enhanced growth of the cell line MDA-MB-453 which has the same biomarker phenotype as TNBC, is known to be ER-independent and AR-dependent [93]. Intriguingly, 10%–35% of TNBC express androgen receptors [94]. Moreover, it has been suggested that a subset of TNBC cases may benefit from the addition of androgen blockade to their therapy [95]. Bicalutamide, a nonsteroidal competitive androgen inhibitor, has been used in the treatment of advanced prostate cancer, but until recently [96], its anticancer effects had not been clinically tested in women. Specifically, the NCT00468715 is an ongoing clinical trial evaluating the use of bicalutamide in the treatment of 65 women with ER/PR-negative, AR-positive metastatic breast cancer with results presented at the 2009 ASCO Breast Cancer Symposium. Clinically, bicalutamide was well-tolerated and preliminary analyses have demonstrated disease stabilisation in ER/PR negative, AR positive tumours with AR inhibition [96].

3.8. HDAC inhibitors

Other possible novel targeted therapies for TNBC include HDAC (histone deacetylase inhibitors) inhibitors, such as vorinostat which suppress cancer-cell proliferation by inducing cell-cycle arrest and/or apoptosis [97]. A paper published in Cancer Letters discusses vorinostat-induced apoptosis in the TNBC cell line MD-MBA-231 and the role of the p38 MAP kinase in the suppression of cancer growth. Interestingly, knockdown of p38 MAP kinase was associated with decreased caspase-3 cleavage. This is of note as cleaved caspase 3 plays a role in autophagy by regulating the extracellular export of autophagic vacuoles; an alternative cellular fate [98,99].

3.9. Immunotherapies and vaccines

TNBC patient outcome has been shown to correlate with the presence of a tumour-immune infiltrate which suggests an area of great potential for effective TNBC treatment [30]. Tumour-infiltrating lymphocytes (TILs) play a role in controlling the clinical progression of various epithelial cancers [100] with a recent review discussing the potential role that the immune microenvironment plays in breast cancer development [101].

The presence of an intense lymphocytic infiltrate may predict response to neoadjuvant chemotherapy in breast cancer [102]. This was proposed following an analysis of core biopsies from patients enrolled in the GeparDuo and GeparTrio trial. In particular, the presence of intratumoural lymphocytes and lymphocyte-predominant breast cancers were associated with a 31% and 41% pathological complete response (pCR) rates, respectively. In comparison, the pCR rates were only 2% in patients without any lymphocytic infiltration [102]. At the San Antonio Breast Cancer symposium in December 2013, Denkert et al., presented findings from the GeparSixto trial (GBG 66), where increased TILs predicted benefit from addition of carboplatin to neoadjuvant therapy for TNBC and HER2 positive breast cancer [103]. At the same session, Adams et al., demonstrated the prognostic value of TILs in two phase II randomised adjuvant breast cancer trials: Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 [104].

Given that TNBC has been recently categorised based on 6 molecular subtypes [30], the immunomodulatory (IM) subtype, characterised by elevated expression of genes involved in T-cell function, immune transcription, interferon (IFN) response and antigen processing, could benefit from immunotherapies. This would be a novel treatment strategy that may be efficacious to this subtype of TNBC. Interestingly, the immunomodulatory subtype has been shown to overlap with medullary breast cancers, with gene expression profiling showing that medullary breast cancer is a subgroup of basal breast cancer displaying a prominent lymphocytic reaction that is associated with a favourable prognosis [105,106].

Immune-checkpoint blocking antibodies and immune-stimulating therapies might act synergistically when combined with chemotherapeutic drugs. The adoptive transfer of chimeric antigen receptor (CAR) -engineered T cells and tumour vaccines against cancer-testis (CT) antigens, which appear highly expressed in TNBC as a result of epigenetic changes, are also novel therapeutic strategies for the TNBC immunomodulatory subtype [105].

The more aggressive claudin-low subtype often presents with an intense immune cell infiltrate and stem cell features with epithelial-mesenchymal transition features [107]. This subtype could therefore also potentially be targeted by immunotherapy treatment [108].

3.10. Other novel signalling pathways

New sequencing technologies have also identified promising therapeutic targets that alter apoptotic pathways. In one case, the developmental signalling pathways Wnt/ β -catenin, NOTCH and Hedgehog have recently been shown to play an important role in

the pathogenesis and progression of TNBC with new potential therapeutic approaches inhibiting these pathways published [109].

JAK2 amplifications in TNBCs has also been shown to be associated with a worse patient outcome and appear to be enriched in TNBCs. Balko et al., at the 2013 San Antonio Breast Cancer Symposium presented these findings and demonstrated that after neoadjuvant chemotherapy, TNBCs enriched with this amplification were associated with a poor prognosis, potentially identifying JAK2 inhibitors as novel and potential treatments for TNBC [110]. Table 1 summarizes the current novel therapies for TNBC.

3.11. N-acetyl cysteine (NAC)

It has been advocated that new cancer therapies should be developed to target the metabolic inter-dependencies between epithelial cancer cells and their surrounding stromal microenvironment. Moreover, it has been suggested that cancer chemo-prevention and treatment should focus on the development of new powerful anti-oxidants to minimise oxidative stress, L-Lactate production and tumour stromal co-evolution.

The thiol anti-oxidant N-acetyl cysteine (NAC) behaves as an anti-inflammatory due to NF κ B inhibition and inhibits L-Lactate production. Due to the fact that lactate has been shown to decrease after chemotherapy or radiotherapy in animals [225], the monitoring of this metabolite may be predictive of therapeutic response [226]. Clinically, N-acetyl cysteine (NAC) is already used in clinical practice as an antidote for acute drug intoxication, in particular as a result of Paracetamol poisoning with safe dosages and careful assessments of its side effect profile already known even at very high doses for long-term treatments [227–229]. It also has a clinical role in inflammation and acute respiratory distress [230].

However, the potential therapeutic use of NAC as an anti-cancer agent is yet to be tried out in the clinical setting. Intriguingly, its ability to negatively impact on the ability of cells to undergo senescence and or autophagy and indeed resistance to anoikis, key cellular fates induced in hypoxic environments, identifies it as an attractive candidate to be used in combination with our established adjuvant and neo-adjuvant treatments in TNBC, an established and recognised hypoxic tumour with an innately chemoresistant biology. Tables 2a and 2b summarise the evidence to date that NAC could have a role as an antioxidant in cancer and more specifically in TNBC.

4. Summary

Women presenting with triple negative breast cancer (TNBC) are treated in the most part similarly in the adjuvant and neoadjuvant

Table 1
Summary of novel therapies for TNBC.

1. DNA Repair Mechanisms:
PARP Inhibitors – Olaparib, Iniparib, Veliparib
Platinum Salts – Carboplatin, Cisplatin
2. Non-Taxane Microtubule Stabilising Agents:
ixabepilone
Eribulin
3. Angiogenic Inhibitor
Anti-VEGF Monoclonal Antibody – Bevacizumab (Avastin®)
4. EGFR/P13K/AKT/mTOR Signalling Pathways:
Anti-EGFR Monoclonal Antibody – Cetuximab
EGFR Tyrosine Kinase Inhibitors – Dasatinib, Neratinib, Sunitinib
mTOR Inhibitor – Temsirolimus, Everolimus, Deforolimus
5. Androgen Receptor inhibition
Bicalutamide
6. Histone deacetylase inhibition (HDAC)i
Vorinostat
7. Immunotherapies & Vaccines
8. Other Novel Signalling Pathways:
Hedgehog – monoclonal antibodies, small molecular inhibitors
NOTCH – monoclonal antibodies
WNT/ β -catenin signalling – monoclonal antibodies, ligand receptor inhibitors

setting to women who present with non-triple negative breast cancer. This is despite the widespread novel therapy strategies that are currently being investigated in clinical trials. Due to varying histological subtypes and molecular profiles within the heterogenous biology that is TNBC, future treatment efficacy and personalised treatment will need to consider the uniqueness of these presentations in order to progress a much needed individualised treatment strategy for these women.

5. Chemoresistance in TNBC

Chemoresistance can be attributable to the fact that although cytotoxic chemotherapy aims to kill cancer cells through apoptosis, tumour cells have the ability to maintain viability following chemotherapeutic exposure by undergoing alternative cellular fates such as cellular senescence, therapeutic induced senescence (TIS) and autophagy.

Tumour environmental stresses such as starvation, hypoxia and DNA damage have been shown to induce such fates as autophagy [111] and senescence [112]. Both cellular fates being significantly associated with cancer cell survival and chemoresistance [113,114].

Genomic acknowledgment of at least 5 intrinsic breast cancer subtypes coupled with at least 6–7 TNBC molecularly characterised subtypes, makes it not surprising that the task of tailoring treatment for breast cancer presentations a minefield [25,30].

However, adopting the approach of understanding the cellular fates induced in TNBC and using this information to bypass their induction or impede them from survival, has the potential to harness the efficacy of our current therapeutics more effectively. Despite the heterogeneity of TNBC, if we can demonstrate that cellular fates and the tumour micro-environment play a significant role in chemoresistance, a potential treatment target could be exploited for this tumour type by targeting cellular fates and not specific signalling pathways.

5.1. Established mechanisms of chemoresistance in TNBC

Standard chemotherapy remains the backbone of systemic triple negative breast cancer (TNBC) treatment even amidst advances in cancer treatment as neither hormonal therapy nor anti-HER2 agents are efficacious against TNBC due to the absence of a target [73]. At present, the cure for cancer continues to escape oncologists due in a large part to chemoresistance, which accounts for 90% of drug failures in metastatic cancers [115]. Fig. 1 demonstrates six mechanisms of chemoresistance in TNBC that are discussed in detail in this section namely, 1) APC transposrters, 2) β -tubulin III, 3) mutations in DNA repair enzymes such as topoisomerase II and DNA mismatch repair enzymes, 4) alterations in genes involved in apoptosis, 5) ALDH1 and glutathione (GSH)/Glutathione-S-transferase (GST) and 6) NF- κ B signaling pathways.

5.1.1. ABC transporters

One proposed method of chemoresistance involves ATP-binding cassette (ABC) transporters found in a subpopulation of cancer stem cells (CSCs), called side population (SP) cells [116]. Efflux of chemotherapeutic drugs by SP cells has been linked to the ABC transporter

Table 2b

Evidence that N-acetyl cysteine (NAC) has a role in targeting TNBC viability.

1. In comparison to the non-malignant breast cell line MCF-10A, it has been shown in the TNBC cell line MB-MDA-231 *in vitro*, that redox controls progression of the cell cycle suggesting that loss of redox control could drive aberrant cancer cell proliferation [235]. Indeed redox regulation of cancer cells is emerging as a potential therapeutic target in cancer [152].
2. Treatment of the TNBC cell line MDA MB 435 with NAC results in apoptosis and reduction of microvascular density within the core of the tumour leading to significant tumour cell apoptosis/necrosis. This study demonstrated that NAC promotes anti-angiogenesis resulting in endothelial apoptosis and vascular collapse in the tumour [236].
3. NAC appears lethal in the BRCA1-deficient TNBC cell lines HCC 1937 and MDA MB 231. Immunostaining for MCT4 (a functional marker of hypoxia, oxidative stress, aerobic glycolysis, and L-lactate efflux) [237] could be used as a cost-effective biomarker to monitor the response to antioxidant therapy [238].

expressed in these cells. Three ABC transporters in particular have been extensively studied in TNBC namely (a) multidrug-resistant protein-1 (MRP1) which confers resistance to agents such as vinca alkaloids, anthracyclines, and high-dose methotrexate but not paclitaxel or mitoxantrone [117], (b) breast cancer resistance protein (ABCG2) [118–120] which is responsible for the efflux of drugs such as doxorubicin, and (c) the P-glycoprotein (MDR1) pump which pumps a wide array of chemotherapeutics out of cancer cells, including paclitaxel [115]. Specific inhibitors of ABC transporters are currently being tested as a means for overcoming this chemoresistance along with non-specific methods such as nanodiamond conjugation of chemotherapeutic drugs to impair efflux through MDR1 overexpressing cells.

5.1.2. Overexpression of β -tubulin III subunit induces paclitaxel resistance

Tomassi et al., 2007 and Paradiso et al., 2005 have found that in advanced breast cancer, β III overexpression correlated with paclitaxel resistance and progression of disease in patients given first-line paclitaxel chemotherapy [121,122].

5.1.3. Mutations in DNA repair enzymes and enzymes altering drug sensitivity

Reduced levels of expression or function of topoisomerase II, an enzyme critical in DNA replication and repair, have been shown to lead to chemoresistance to drugs such as the anthracyclines and epipodophyllotoxins [123,124]. Chemoresistance may also arise from aberrant DNA mismatch repair enzymes that function to repair DNA damage from various chemotherapeutics such as alkylating agents, platinum compounds, and anthracyclines. Fedier et al., 2001 have further shown that there is a correlation between the loss of DNA-mismatch repair proteins MSH2 and MLH1, and the associated micro-satellite instability genotype coupled with resistance to topoisomerase II inhibitors (epirubicin, doxorubicin, and mitoxantrone [125].

5.1.4. Alterations in genes involved in apoptosis

Alterations in genes regulating apoptosis (p53, caspase-3 s, bcl-2, bcl-x) have been linked with chemoresistance to various chemotherapeutics such as cyclophosphamide, doxorubicin, methotrexate, fluorouracil, and tubulin inhibitors [126–130].

5.1.5. Drug inactivation/detoxification as a mechanism of chemoresistance

Overexpression of ALDH1A1 and ALDH3A1 have been shown to result in greater inactivation of cyclophosphamide leading to chemoresistance [131]. Moreover, increased glutathione and glutathione-S-transferase activity results in increased breakdown of alkylating agents and cisplatin thereby increasing resistance to these compounds [132]. Lastly, polymorphisms in the cytochrome P450 system (namely CYP3A4 and CYP2C8) have been associated with highly basal enzymatic activity which may also contribute to chemoresistance [133].

Table 2a

Evidence that N-acetyl cysteine (NAC) can act as an anti-cancer agent.

1. NAC induces p53-dependent apoptosis, has anti-tumour activities and has been shown to be cancer chemo preventive in clinical studies, with promise in preventing tumour progression [229,231].
2. Treatment with thiol-containing antioxidants (such as NAC) has the potential to preferentially induce apoptosis in preneoplastic and neoplastic human lung fibroblasts cells [232].
3. NAC is involved in the downregulation of VEGF expression, by limiting hypoxia-induced transcription via hypoxia inducible factor-1- α (HIF1- α) and repression of reactive oxygen species (ROS) [233]. Clinically this has resulted in angiogenesis inhibition and thus reduction in tumour growth in Kaposi Sarcoma [234].

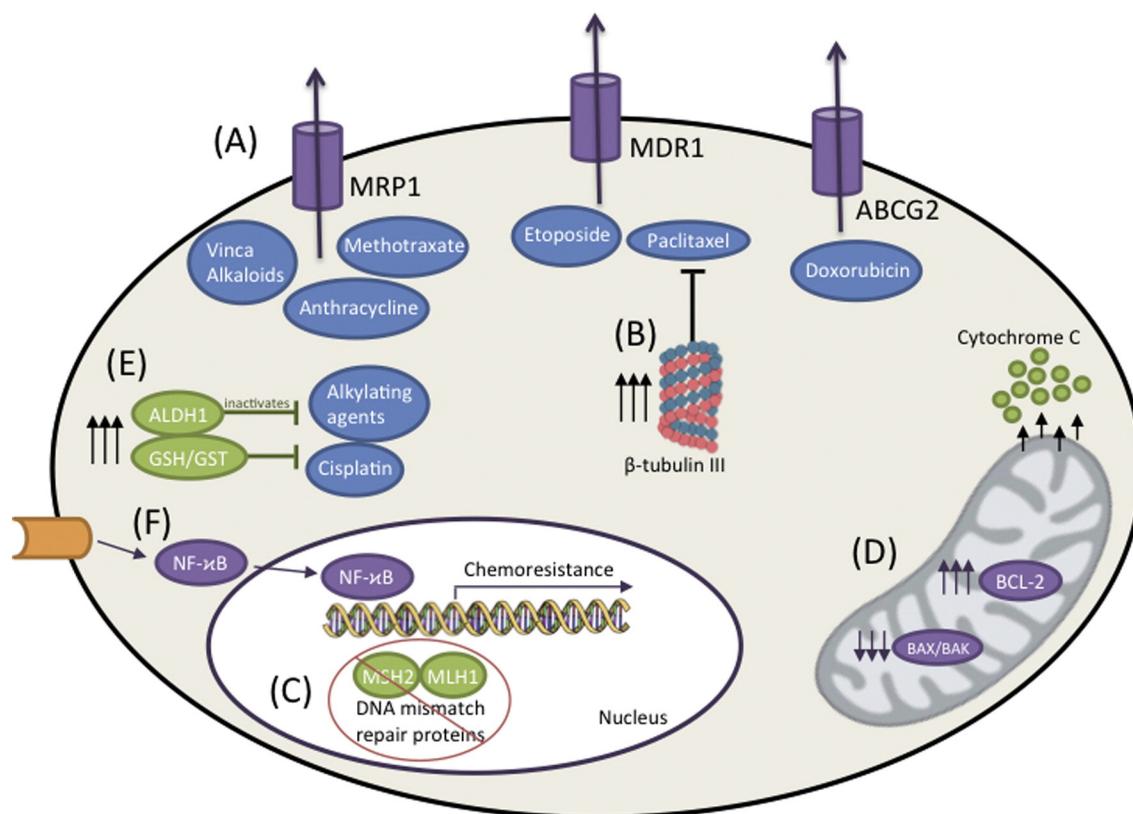


Fig. 1. Mechanisms of Chemoresistance in TNBC. (A) ABC transporters efflux chemotherapeutics out of cancer cells (B) Overexpression of β -tubulin III subunit induces paclitaxel resistance (C) Mutations in DNA repair enzymes and enzymes altering drug sensitivity (D) Alterations in genes involved in apoptosis prevent chemotherapy-induced apoptosis (E) ALDH1 and glutathione (GSH)/Glutathione-S-transferase (GST) mediate chemotherapeutic inactivation/detoxification (F) Role of NF- κ B signaling pathway in chemoresistance.

5.1.6. Role of the NF- κ B signaling pathway in chemoresistance

Treatment of breast cancer with disulfiram and copper sensitises breast CSCs to paclitaxel possibly by inhibiting constitutively active NF- κ B thereby alluding to the correlation between aberrant NF- κ B regulation and chemoresistance in TNBC [134].

5.1.7. KIF14-mediated AKT phosphorylation promotes chemoresistance in TNBC

Singel et al., 2014 published that overexpression of KIF14 increased chemoresistance to docetaxel in 34 cases of locally advanced TNBC. Conversely KIF14 knockdown and chemical inhibition resulted in lower levels of AKT phosphorylation and activity, leading to significant chemosensitisation when cells were treated with docetaxel. Importantly, phosphorylated AKT (pAKT) is an anti-apoptotic protein kinase in the well-characterised prosurvival PI3K/Akt signaling pathway [135].

5.1.8. Molecular markers that could predict resistance to chemotherapy in TNBC

Chekhun et al., 2009 have found that overexpression of metallothioneins and glutathione-S-transferase were associated with resistance to platinum salts while Zhou et al., 2011 reported that low expression of the eukaryotic translation initiation factor 4E (eIF4E) increased sensitivity to platinum salts [136,137]. As for taxane chemotherapy, overexpression of the microtubule-associated protein 2 (MAP2), interleukin 6 (IL6), matrix metalloproteinase 9 (MMP-9), and low expression of inhibitor of tissue-plasminogen activation (t-PAI) increased sensitivity [138,139] while overexpression of GRB-7 protein, alpha B-crystallin, and P-glycoprotein conferred a chemoresistant phenotype [140,141]. Resistance to PARP inhibitors was increased by the overexpression of the multi-drug resistance efflux pumps (MDR 1,2) [142] and decreased by a deficiency of BRCA-1, BRCA-2, XRCC2,

and XRCC3 genes [143]. Conversely, the restoration of function of the tumour suppressor gene BRCA-1 led to an increased chemoresistance against PARP inhibitors [142].

Lastly, the low expression of the zinc-finger enhancer binding protein ZEB-1 increased sensitivity of TNBC cells to bicalutamide [144].

5.2. Selective resistance of cancer stem cells (CSCs) to chemotherapy

Treatment of TNBC with chemotherapeutics such as taxanes is initially very effective in most patients. However, the majority of these tumours will recur following chemotherapy treatment [145]. These recurrent tumours are typically chemoresistant, and are associated with a poor prognosis [146]. There is an increasing amount of evidence that suggests that following treatment, a small population of cells survive, with stem-like properties that may be responsible for tumour recurrence [147]. These cells have been termed cancer stem cells (CSCs). CSCs are tumour cells with self-renewal properties that have the ability to form a recurrent chemoresistant tumour. Lapidot et al. provided early evidence in 1994 of the existence of CSCs by identifying a subpopulation of cells in acute myeloid leukaemia (AML) that were capable of initiating leukemic growth when introduced into severe combined immunodeficiency (SCID) mice [148].

Recent evidence suggests that chemoresistant populations of cancer stem cells may be responsible for relapse in triple negative breast cancer (TNBC). The TGF- β family of cytokines has been implicated in CSCs. TGF- β 1 and the TGF- β type 1 receptor (TGF- β R1) have been shown to be over-expressed in a subpopulation of breast cancer cells with CSC features. TGF- β induces epithelial-to-mesenchymal transition (EMT) in mammary cells, which has been associated with tumour stem-like properties in cells [149].

In 2013, Bhola et al. reported that following chemotherapy treatment, TNBC biopsies showed an increase in RNA transcripts of genes

associated with CSCs and TGF- β signalling. Treatment with paclitaxel also increased TGF- β signalling and CSC properties in TNBC cell lines and mouse xenografts [150]. This suggests that initial treatment may form a subpopulation of cancerous cells with stem-like properties that can regenerate a chemoresistant tumour.

However, Bhola et al. also reported that using the TGF- β type 1 receptor kinase inhibitor LY2157299 in combination with paclitaxel, prevented re-establishment of tumours in TNBC xenografts in mice [150].

In 2014, Samanta et al. published that treatment of human TNBC cell lines with paclitaxel or gemcitabine resulted in an increase in hypoxia-inducible factors (HIFs) expression and transcriptional activity. This resulted in an enrichment of the CSCs population through interleukin-6 and interleukin-8 signalling [151]. This indicated that HIFs may be involved in acquired chemoresistance of breast cancer stem cells.

This study also indicated that the administration of HIF inhibitors in combination with paclitaxel or gemcitabine, overcame the CSCs resistance to the chemotherapeutics, which lead to tumour eradication, both *in vitro* and *in vivo* [151].

Both these studies show evidence that the treatment of TNBC with current therapeutics may result in the formation of a subpopulation of chemoresistant cancer cells with stem-like properties, that if not eliminated are free to form a tumour that is not responsive to current chemotherapeutics.

5.3. Tumour hypoxia's role in TNBC chemoresistance

Breast cancer cells are subjected to a high level of oxidative stress, both intra- and extra cellularly [152]. Moreover, it is known that TNBC is associated with a more hypoxic phenotype possibly due to activation of the mTOR pathway and HIF-1 α stabilisation [153]. Fig. 2 is a referenced illustration demonstrating the role that hypoxia plays in cellular fates. Clinically, the fact that TNBC's are amenable to fluroeoxyglucose-positron emission tomography (FDG-PET) scanning as a result of the "Warburg effect" [154] also highlights their more hypoxic phenotype (Table 3).

The term hypoxia describes the phenomenon of an inadequate supply of oxygen as a result of cells outgrowing their vasculature [155] beyond the size of several mm³. This compromises the cells biological functions with a concomitant increase in compensatory glycolysis [156]. Importantly, hypoxia is a key microenvironment associated with the tumour milieu with this acidic setting compromising chemotherapeutic efficacy due to the varying levels of oxygen present in the tumour [157]. Moreover, a hypoxic tumour environment results in an increase in cellular senescence [114,159] and therefore potentially propagating a chemoresistance phenotype.

Clinically, evidence that TNBC tumours are more hypoxic than non-TNBC histologies is presented in Table 3 by either an increased FDG uptake and standardise uptake values (SUV) on PET/CT imaging or increased Carbonic Anhydrase IX (CAIX) immunohistochemical assessment on full face paraffin embedded (FFPE) TNBC clinical material. TNBCs are amenable to FGP-PET scanning on account of the "Warburg effect" [154] with one study addressing the use of ¹⁸F-FDG PET/CT as an early assessment in TNBC during neoadjuvant chemotherapy to identify patients who are unlikely to achieve a pathologic complete response and are at a high risk of early relapse [160]. CAIX, is an accepted marker for tissue hypoxia and has been shown to be associated with a worse prognosis for patients with TNBC [161,162]. Recent evidence shows that basal endoplasmic reticulum stress (ERS) is typically activated in TNBC and cooperates with hypoxia signalling to promote tumour progression and relapse [163].

5.4. Cellular senescence

The term cellular senescence was first discovered and the term coined in aging human fibroblasts by Hayflick and Moorhead in 1961 who observed an irreversible exit of the cells from the cell cycle. Senescent cells, however, although initially having lost their proliferative capacity, maintain cellular viability and metabolic activity [112] generating a senescent secretome called the SASP phenotype or senescence associated secretory phenotype.

In relation to the role of cellular senescence in cancer, on the one hand it can be regarded as a tumour suppressor mechanism for cells

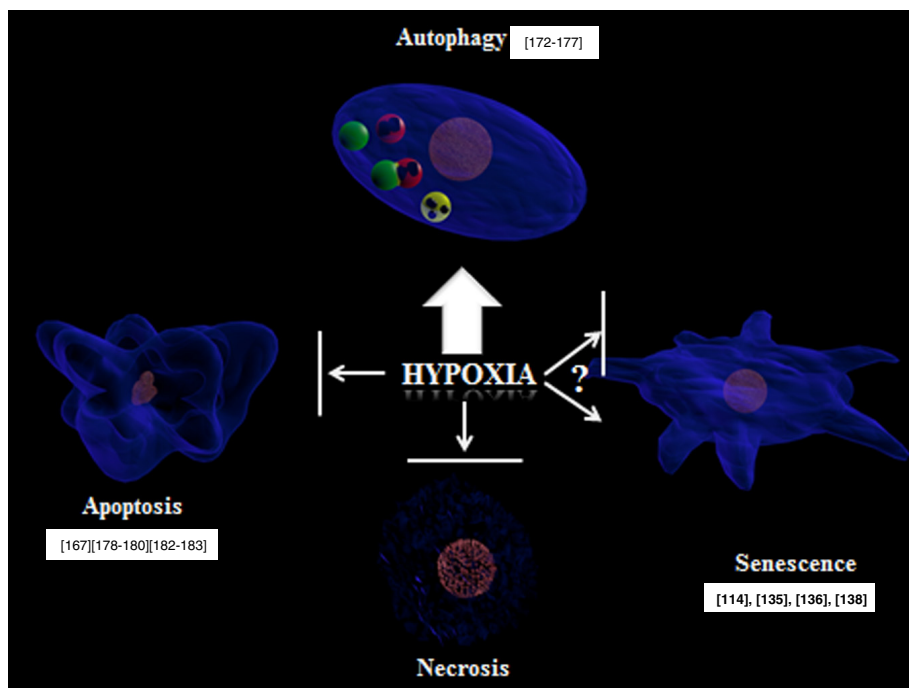


Fig. 2. The role hypoxia plays in cellular fates including references.

Table 3

Clinical published evidence that triple negative breast cancer (TNBC) is more hypoxic than non-TNBC histologies.

Hypoxia Appreciation	Patient Numbers	Details	Reference
FDG-PET	N = 88 TNBC = 29 ER +/PR +/HER2- = 59	TNBC tumours associated with enhanced FDG uptake, and detected with very high sensitivity using FDG-PET imaging.	Basu et al., 2008 [154]
FDG-PET	N = 41 TNBC = 22 Non-TNBC = 19	The degree of tumour FDG uptake correlated significantly with proliferation in women with TNBC, suggesting a potential role of FDG-PET in monitoring treatment response for this group of women.	Tchou et al., 2010 [167]
FDG PET/CT	N = 78 (Stage II / III Breast Cancers)	FDG-PET/CTs were acquired before and after the first cycle of chemotherapy to evaluate early metabolic response. In TNBC, the pre-chemotherapy standardised uptake value (SUV) was higher than in non-TNBC.	Keam et al., 2011 [168]
18 F-FDG PET/CT	N = 152 (Primary Breast Cancers)	High SUV levels were significantly correlated with tumour size, poorer grade, nuclear atypia, increased mitotic index, negative hormone receptor status, high score of HER-2 expression, lymph node metastasis, and IDC in comparison with invasive lobular carcinoma.	Ueda et al., 2008 [169]
18 F-FDG-PET	N = 91 (Invasive Ductal Carcinomas)	High FDG uptake correlated with poor prognosis factors, such as tumour invasiveness >2 cm, higher tumour grade, hormonal receptor negativity, and triple negativity.	Kim et al., 2012 [170]
18 F-FDG-PET	N/A	18 F-FDG-PET had a higher sensitivity for TNBCs than oestrogen-positive and human epidermal growth factor receptor 2-positive tumours.	Dogan et al., 2012 [171]
Protein expression of CAIX and BRCA1 evaluated by AQUA (Automated Quantitative Analysis) Technology (IHC)	N = 619 TNBC = 111 Unselected Cohort = 508	CAIX was expressed in 7% of the unselected breast cancer cohort and in 25% of the TNBCs and significantly associated with the triple negative phenotype. CAIX also associated with poor outcome.	Neumeister et al., 2012 [161]
FFPE Tissue Sections stained for various hypoxic markers, including; HIF-1 α , PHD1-3, and CAIX	CAIX Staining: N = 456 Basal-like = 62	50% of basal-like tumours expressed HIF-1 α , and more than half expressed at least one of the PHD enzymes and FIH-1. Basal-like tumours were nine times more likely to be associated with CAIX expression. Patients with any CAIX-positive breast tumour phenotype or CAIX basal tumours had a significantly worse prognosis than CAIX-negative tumours, partly attributable to an enhanced hypoxic response.	Tan et al., 2009 [162]

Note:- FDG-PET = fludeoxyglucose-positron emission tomography, SUV = standardised uptake value, SUV represents the ratio of the image derived radioactivity concentration found in a selected part of the body at a certain time point. CAIX = carbonic anhydrase, HIF-1 α = hypoxia inducible factor, IDC = invasive ductal carcinoma, PHD = prolyl hydroxylase domain, FIH = factor inhibiting hypoxia-inducible factor.

in response to DNA damage, telomere attrition, oxidative stress, DNA damage and other tumour-promoting insults [112] and indeed there is evidence that the progression of malignant invasive tumours is believed to arise from the evasion of senescence in the pre-invasive precursor tumour [164]. Recent evidence however also implicates the senescence pathway in breast carcinogenesis [165] through promoting tumour progression by stimulating growth and transformation in adjacent cells through the senescence associated secretory phenotype (SASP) [166].

5.5. Hypoxia, senescence and stroma

Table 4 demonstrates the role hypoxia plays in determining cellular senescence in cancer with clear conflicting views evident.

The contribution of the stromal component and the interdependencies between tumour epithelial cells (TECs) and their stromal environment, tumour associated fibroblasts (TAF) in an oxygen stressed /L-lactate rich environment has been identified as a key cancer research area. This is new model of cancer metabolism, identifying the role of aerobic glycolysis and L-lactate production in fuelling tumour growth and metastasis [137].

Intriguingly, senescent cells produce considerable amounts of lactate and play a key role in the tumour-stroma co-evolution. In relation to the relevance of this to breast cancer, lower levels of lactate have been observed in breast cancer patients with long-term survival (≥ 5 years, survivors) compared to patients who died of cancer recurrence [174].

This novel hypothesis led by Professor Lisanti in Thomas Jefferson University, Philadelphia is coined the “Reverse Warburg effect” as aerobic glycolysis takes place in stromal fibroblasts, rather than epithelial cancer cells. This group demonstrated that MCT4 immunostaining of human TNBC micro arrays can be used to directly assess prognosis [175]. MCT4 is a functional marker of hypoxia, oxidative stress, aerobic

glycolysis, and L-lactate efflux. High stromal MCT4 levels were associated with decreased overall survival (<18% survival at 10 years post-diagnosis). In contrast, patients with absent stromal MCT4 expression had 10 year survival rates of ~97%. Interestingly, epithelial MCT4 staining demonstrated no prognostic value, suggesting that the tumour stroma may represent a needed focus for targeted therapy.

Oxidative stress induced by the tumour epithelial cells (TECs), results in increased levels of fibronectin (FN) on the surface of adjacent senescent tumour associated fibroblasts (TAFs) [176]. Increased production of FN is one of the most typical features of senescent cells [177,178] and potentially is an antigenic determinant unique to cellular senescence [179]. Intriguingly FN is differentially upregulated in TNBC compared to Her2 +, ER-PR- [180]. This increased synthesis of FN is mediated by TGF- α 1 [176] which can stimulate p16 (INK4), a marker of cellular senescence [181].

Mechanistically, reconstitution of p16 has been shown to increase the expression of the FN receptor $\alpha 5 \beta 1$ on TECs [182]. Moreover, elevated intracellular reactive oxygen species (ROS) produced by oxidatively stressed mitochondria increases $\alpha 5 \beta 1$ expression on tumour epithelial cells (TECs) [183]. When bound to extracellular matrix components such as FN, $\alpha 5 \beta 1$ co-operates with growth factors to activate anti-apoptotic signalling.

It has also been shown that with increasing age, the accumulation of senescent cells alter the tissue architecture to the extent that it provides a permissive environment of uncontrolled growth of adjacent premalignant cells [184]. Moreover, fibroblasts adjacent to malignant tissue in contrast to those neighbouring the normal epithelium have been found to be senescent [185] suggesting a possible role for their assessment in identifying tumour free margins.

The role of epithelial versus stromal features of senescent cells in predicting outcome for patients presenting with TNBC, irrespective of the chemotherapeutic used or the individual subtype of TNBC, is a potential area of research.

Table 4
The role hypoxia plays in determining cellular senescence in cancer.

Cellular Fate	Determination of Cellular Fate	Details/Results
Senescence Watson et al., 2009 [114]	SA- β -gal	PwR-1E benign prostate epithelial cells and equivalently aged hypoxia-adapted PwR-1E cells were used to identify the phenotypic and epigenetic consequences of chronic hypoxia in prostate cells. The impact of chronic hypoxia (1% O ₂) on cellular senescence was assessed by β -galactosidase activity for both cell lines. Increased levels of cellular senescence were identified in the hypoxic population of PwR-1E cells compared to their normoxic counterparts.
Senescence Hammond et al., 2005 [172]	p53 Immunoblotting	RKO, 293 T, RCC4, and HCT116 cell lines used. HIF-1 α protein accumulated with similar kinetics at 0.2% and 2% oxygen. p53 protein accumulated in response to 0.02% oxygen only. p53 accumulated in response to hypoxia independently of HIF-1 α . Accumulation of p53 correlated with replication arrest. Reports conflicting evidence on the role of hypoxia in senescence:
Senescence Welford et al., 2011 [173]	N/A	Excess O ₂ can lead to the production of Reactive Oxygen Species (ROS) which can promote senescence. Reduced O ₂ levels (hypoxia) can reduce the amount of ROS. Hypoxia leads to the stabilisation of HIF, which impacts on many pathways that can affect senescence: HIF binding to Myc can induce p21 expression and inhibit CDC25C, promoting cell-cycle arrest. HIF controls several genes in the senescence-associated secretory phenotype (SASP) that can promote senescence. However, HIF also promotes glycolysis, and can induce expression of TERT, negatively regulating p53, both of which are inhibitory to a senescent phenotype.

Note:- TERT = Telomerase reverse transcriptase, CDC25 phosphatases are important regulators of the cell cycle, Myc = transcription factor.

5.6. Autophagy

Autophagy, type II programmed cell death, is an evolutionarily conserved, self-degenerative process involved in protein and organelle recycling and cellular homeostasis [186]. There are three types of autophagy; macroautophagy [187], microautophagy [188] and chaperone mediated autophagy (CMA) [189]. Macroautophagy involves the formation of a double-membraned structure in the cytoplasm (the autophagosome), which grows and eventually fuses at either end thus capturing the majority of cytoplasm and some organelles in the process. This autophagosome then fuses with the lysosome for degradation [151]. Microautophagy, on the other hand, refers to the direct transfer of cytosolic components into the lysosome by invagination of the lysosomal membrane and subsequent budding into vesicles within the lysosome itself [152]. Chaperone mediated autophagy (CMA) uses a different approach completely with specific selection and removal of proteins with particular peptide sequence motifs that are recognised by molecular chaperones, which then fuse with the lysosome via a lysosome-associated membrane protein Lamp2a for selected removal [151].

Autophagy appears to modulate both cell viability and death [190]. However, the role of autophagy in cell death is controversial. The presence of autophagic vacuoles in dying cells may be interpreted in one of two ways: either cells activate autophagy in an attempt to survive, or autophagy is a part of the process of cell death [191].

Autophagy is often expressed at a basal level in cells representing its role in the recycling of proteins and tissue homeostasis [192]. However, in addition to the role of autophagy in protein quality control, this cellular fate mechanism also plays an integral role in cell survival during cytotoxic stresses such as hypoxia, nutrient deprivation, DNA damage and in response to chemotherapeutics and radiotherapy [193,194].

However, the overall role of autophagy in cancer is far more controversial with its inhibition and induction both showing beneficial and negative effects of tumour cell survival [195–197].

In tumour cells the role of autophagy may depend on the type of tumour, the stage of tumourigenesis and the nature and extent of the insult [198]. Inhibiting cytoprotective autophagy or promoting type II programmed cell death are mechanisms that could enhance the effects of cytotoxic chemotherapeutics [195].

5.7. Autophagy and hypoxia

Autophagy provides the cell with an anaerobic metabolic mechanism which supplies the cell with the basic metabolic portfolio to survive during oxidative stress or nutrient deprivation. It is also responsible for the removal of ROS [199] and unfolded proteins generated during oxidative stress and cellular aging [200].

The role of autophagy in an oncological setting has received much attention, specifically coined as the “Autophagic Tumour-Stromal Model” of cancer metabolism and tumour cell survival [201].

This model explains why angiogenesis inhibitors might not work, and instead induce lethal tumour recurrence, and metastasis. This is partially attributable to the fact that angiogenesis inhibitors drive “hypoxia” in the tumour stromal micro-environment. Hypoxia, in turn, drives oxidative stress and autophagy.

It has already been shown that paclitaxel induces autophagy in the cell line A549 human alveolar adenocarcinoma. However, conversely, inhibiting autophagy potentiates paclitaxel's-mediated apoptotic effects [202].

In vitro studies have also demonstrated that hypoxia protects the TNBC cell line, MDA-MB-231 from paclitaxel-induced apoptosis and concomitantly induced an autophagic response after 2 hours in normoxic and hypoxic condition. Specifically, the mechanism of decreased apoptosis was studied using p62siRNA transfection, a protein involved in autophagosome formation. These results demonstrated that hypoxia diminishes paclitaxel-induced apoptosis in MDA-MB-231 cells via mTOR/JNK pathway [203].

Recently, a new form of autophagy has emerged known as mitophagy, which is believed to play a very important role in cancer cell survival [204], particularly in hypoxic regions of tumours. This is supported by studies of models with monoallelic deletion of Beclin-1 showing increased cell death in these tumour regions [205]. Mitophagy involves the selective removal of damaged mitochondria, subsequently decreasing ROS and cytotoxic molecules of apoptosis and thus, promoting cell survival [206,207]. Mitophagy is induced by BNIP3 whose expression in turn is regulated by hypoxia-inducible factor-1 (HIF-1) expression or the Ras/raf/Erk pathway [207,208]. Ras mutated tumours can therefore become very dependent on autophagic pathways, particularly mitophagy, through overactivation of autophagic pathways such as Ras/Raf/Erk or indirectly through hypoxia-induced expression of HIF-1. Due to the hypoxic nature of tumours, HIF pathways are of great importance in tumour progression. Cells have been found to adapt energy metabolism to hypoxic conditions through 4 main HIF-mediated mechanisms: (1) COX4 subunit switching, (2) inhibition of acetyl-CoA synthesis by activation of PDK1, (3) c-Myc repression and inhibition of mitochondrial biogenesis and the newer concept of

mitochondrial autophagy, which as described above, decreases ROS release from damaged mitochondria thus promoting cell survival [206].

5.8. Autophagy inhibition & TNBC

Protein expression of the autophagy-related microtubule-associated proteins, including beclin-1, light chain (LC) 3A and LC3B have been shown to be the highest in TNBC cells compared to the other breast cancer subtypes, with the lowest expression in the stroma of TNBC [209]. High expression of LC3B is also associated with tumour progression and poor outcome in TNBC [210] demonstrating its role as a potential prognostic marker in TNBC.

Intriguingly cancer cell viability has also been shown to be reduced through induction of non-apoptotic, non-autophagic cytoplasmic vacuolation death in TNBC cells through the expression of the LC3 protein and p62/SQSTM1; a protein involved in autophagosome formation [211].

Moreover, inhibition of autophagy has been identified as a potential adjunctive strategy for enhancing the chemotherapeutic effect of paclitaxel. In addition, by inhibiting autophagy with the pharmacological autophagy inhibitor chloroquine, a synergistic effect of enhanced TNBC cell death both *in vivo* and *in vitro* was noted when used with the endoplasmic reticulum stress aggravator (ERSA) compounds such as the antiretroviral Nelfinavir, and the COX2 inhibitor Celecoxib [212].

Tables 5 and 6 summarise the relationship between hypoxia and the fates of autophagy and apoptosis respectively in TNBC specifically.

6. Conclusion

Finding efficacious treatments for TNBC is critical due to the fact that these women are treated, in the most part with standard chemotherapy. This approach does not tailor to the specific biology of TNBC and its molecular subtypes. Even though the concept of standard chemotherapy in breast cancer is becoming a thing of the past with a) oncotyping and

b) other molecular markers identifying hormone sensitive tumours that gain no benefit from chemotherapy, these do not however, apply to patients with TNBC resulting inevitably in a significant number of women not responding to first line treatment.

With this in mind and in order to improve patient outcome it is crucial to establish a) what factors influence a patients' individualised response to chemotherapy and b) which factors may explain why some patients fail to benefit from standard first-line chemotherapy, while others achieve long-term remission.

In this regard, research specifically focused on interrogating the mechanisms and ability of tumour cells to circumvent and bypass apoptotic death mechanisms through adopting viable cellular fates such as cellular senescence and cytoprotective autophagy is warranted. In particular, targeting these non-apoptotic survival pathways has the potential to enhance the chemotherapeutic efficacy of our current artillery of drugs for women presenting with TNBC.

6.1. Summary

In specifically targeting the survival mechanisms that cancer cells can use to bypass death through apoptosis, viable cellular fates such as cellular senescence and cytoprotective autophagy could potentially be a target for impedance or manipulation and thus increase the death rate of cancer cells. Importantly, both these cellular fates are induced in tumour hypoxia, a key tumour microenvironment associated with TNBC and integral to chemoresistance. In the case of senescence, anti-oxidants such as NAC in combination with standard chemotherapeutics hold potential in eradicating these metabolically active SASP secreting cells, as do the combinatorial use of mTOR inhibitors or glycolytic inhibitors such as rapamycin to promote terminal autophagy. Such approaches are worth serious consideration and may offer more potential than attempts to find specific tailored therapies for the myriad of genetic and epigenetic changes and tumour cell populations unique to each patient's tumour and tumour microenvironment.

Table 5

The role hypoxia plays in determining the cellular fate autophagy.

Pike et al., 2013 [213]	MCF7, MDA-MB-231, HCT116, HEK293, MEF3T6, T47D, and HeLa cell lines	ULK1	Autophagy-initiating kinase ULK1 (UNC51-like kinase 1) is a direct transcriptional target of ATF4 (activating transcription factor 4), which drives the expression of ULK1 mRNA and protein in severe hypoxia. ULK1 is required for autophagy in severe hypoxia and ablation of ULK1 causes caspase-3/7-independent cell death.
Salem et al., 2012 [214]	Human hTERT-immortalized fibroblasts MDA-MB-231 cell	HIF-1α	ULK1 expression is associated with a poor prognosis in breast cancer. shBRCA1 fibroblasts displayed an elevated growth rate. Using immunofluorescence and immunoblot analysis, shBRCA1 fibroblasts demonstrated an increase in markers of autophagy and mitophagy. Most notably, shBRCA1 fibroblasts also displayed an elevation of HIF-1α expression.
Sotgia et al., 2011 [215] Witkiewicz et al., 2011 [216]	Malignant melanoma 44 cases: 24 lymph node metastasis, 20 primary tumours. IHC stained for Cav-1	stromal Cav-1	Using xenografts demonstrated that shBRCA1 fibroblasts induced an approximate 2.2-fold increase in tumour growth when co-injected with MDA-MB-231 cells into nude mice. Coupling of metabolism between epithelial cells and surrounding stroma between oxygenated and hypoxia compartments. Increased autophagy/mitophagy in the stroma drives metabolism in the epithelial cells.
Azad et al., 2008 [217]	Two glioma cell lines (U87, U373). Two breast cancer cell lines (MDA-MB-231, ZR75). One embryonic cell line (HEK293).	BNIP3	Ammonia production in the epithelial cells then drives autophagy in stroma to promote this cycle. Loss of Cav-1 in stroma leads to early recurrence, metastasis, drug resistance and poor clinical outcome.
Tan et al., 2007 [218]	Normal breast tissue n = 11 DCIS n = 81 IDC n = 251	BNIP3 and HIF1a	Under normoxic conditions, all five cell lines displayed etoposide-induced apoptosis whereas hypoxia failed to induce these apoptotic responses. BNIP3 overexpression induced autophagy, while expression of BNIP3 siRNA or a dominant-negative form of BNIP3 reduced hypoxia-induced autophagy. Stained for BNIP3 and hypoxia-inducible factor-1α. BNIP3 expression was significantly up-regulated in the cytoplasm of DCIS and invasive carcinoma compared with normal breast. Nuclear BNIP3 expression was associated with smaller tumour size, low tumour grade (P = 0.005), and oestrogen receptor positivity in invasive tumours. Nuclear BNIP3 expression in DCIS was associated with a 3-fold increase in recurrence and a shorter disease-free survival. No association between HIF-1α and BNIP3 expression.

Note: ULK = autophagy-initiating kinase, a homologue of yeast ATG1, CAV1 = caveolin 1 leads to oxidative stress, BNIP3 = Bcl-2 and nineteen-kilodalton interacting protein-37 and is a member of the Bcl-2 protein family.

Table 6

The role hypoxia plays in determining the cellular fate, apoptosis.

Cellular model	Details	Reference
MDA-MB-231 Breast cell line	Hypoxia protected MDA-MB-231 cells from paclitaxel-induced apoptosis and concomitantly induced autophagy response after 2 hours in normoxic and hypoxic condition. The mechanism of decreased apoptosis was studied using siRNA and showed that hypoxia decreases the apoptotic response via mTOR and JNK activation.	Notte et al., 2013 [203]
MCF7 Breast cell line	Hypoxia decreased paclitaxel-induced apoptosis and G2/M arrest was visualised by MTT assay and flow cytometry. Also, hypoxia diminished paclitaxel-dependent polymerisation of tubulin. Interestingly it was found that under hypoxic conditions, Cyclin B1 was down-regulated which lead to decreased apoptotic death. Overexpression of Cyclin B1 in MCF7 cells partially restored sensitivity to paclitaxel in hypoxic cells.	Dong et al., 2012 [219]
A2780 Ovarian cell line	Human ovarian xenograft tumours (A2780) in mice were severely hypoxic. Phosphorylation of protein STAT3 was observed under hypoxia and knock-down experiments revealed that STAT3 might be an important protein in mediating resistance to cisplatin and paclitaxel under hypoxic conditions.	Selvendiran et al., 2009 [220]
MDA-MB-231 Breast cell line Du-145 Prostate cell line	Hypoxia induces resistance to etoposide in breast and prostate cancer due to the prevention of DNA damage and strand breaks after exposure. Cell treatment with small interfering RNA targeting hypoxia-inducible factor 1 prevented the hypoxia-induced decreases in topoisomerase II α levels, abolished the protective effect of hypoxia against etoposide-induced DNA damage, and inhibited hypoxia-induced etoposide resistance.	Sullivan et al., 2009 [221]
C13K Ovarian cell line	Ovarian cancer C13K cells under hypoxic conditions are resistant to cisplatin treatment. Inhibition of HIF1 α transcriptional activity by the small molecule – noscapine improves sensitivity to cisplatin.	Su et al., 2011 [222]
MDA-MB-231 Breast cell line	Hypoxia protected MDA-MB-231 breast cancer cells against paclitaxel-induced apoptosis. The implication of HIF-1 and AP-1 in the hypoxia-induced anti-apoptotic pathway was investigated by the use of specific siRNA. Results showed that both transcription factors are important in mediating resistance.	Flamant et al., 2010 [223]
MDA-MB-468 MCF-7 HCC1937 Breast cell lines	Hypoxia induced change in BRCA1 subcellular localisation to the nucleus without a change in the overall protein levels. Hypoxia also induced TRAIL expression on the surface of the cell which increased apoptotic response to apoptotic stimuli. BRCA1 mutant cells were defective in apoptotic activation under hypoxic condition compared to BRCA1 wt cells.	Fitzgerald et al., 2007 [224]

Note: JNK = c-Jun N-terminal kinase, STAT3 = Signal transducer and activator of transcription 3, TRAIL = TNF-related apoptosis-inducing ligand, TNF = tumour necrosis factor, wt = wild type.

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